

Flow Cytometric Detection of Reactive Oxygen Species

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[Abstract] Reactive oxygen species (ROS) are molecules containing hydroxyl radicals or peroxides with unpaired electrons. In healthy aerobic cells, ROS are produced naturally as a byproduct of oxidative phosphorylation, oxidoreductase enzymes, or metal catalyzed oxidation at a controlled rate. However, ROS can be induced under some stress conditions especially exposure to environmental oxidants and certain drugs that leads to oxidative stress. Exceed ROS can cause damages in the building blocks of cells including DNA, proteins, and lipids, and eventually results in cell death. Cell-permeant 2', 7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) is a widely used ROS indicator. The reduced non-fluorescent fluorescein H₂DCFDA can be oxidized and converted into fluorescent 2', 7'-dichlorofluorescein (DCF) by intracellular ROS. In this protocol, we applied H₂DCFDA to label the intracellular ROS and detected the DCF intensity by flow cytometry.

Materials and Reagents

1. Cells to analyze (this protocol has been successfully performed on A549, CL1-0, and IMR-90 cells)
2. Dulbecco's Phosphate-buffered saline (DPBS)
3. 2',7'-dichlorofluorescein diacetate (H₂DCFDA) (Life Technologies, Invitrogen™, catalog number: D-399)
4. Anhydrous DMF (N,N-dimethylformamide) (Sigma-Aldrich, catalog number: 227056)
5. N-acetyl-L-cysteine (NAC) (Sigma-Aldrich, catalog number: A7250)
6. Hydrogen peroxide (H₂O₂) (Sigma-Aldrich, catalog number: 349887)
7. 5 ml polystyrene BD falcon round-bottom tube with cell strainer cap (BD Biosciences, catalog number: 352235)

8. Sodium Chloride (NaCl)
9. Potassium Chloride (KCl)
10. Potassium Phosphate, monobasic (KH_2PO_4)
11. Sodium Phosphate, dibasic (Na_2HPO_4)
12. DPBS (see Recipes)
13. H_2DCFDA stock (10 mM) (see Recipes)
14. NAC stock (1 M) (see Recipes)
15. H_2O_2 stock (1 M) (see Recipes)

Equipment

1. Flow cytometry
2. Water bath
3. Centrifuge

Procedure

1. Cells were cultured with complete medium in a 6-cm dish at 37 °C and 5% CO_2 .
2. Cells were treated with NAC or H_2O_2 after reaching 70-90% confluency. ROS scavenger NAC is a precursor to cysteine and glutathione which are strong antioxidants; while H_2O_2 is a compound with an oxygen-oxygen single bond and is known as a strong oxidizer.
3. (For negative control) Incubate cells with freshly prepared 5 mM NAC in culture medium for 1 h at 37 °C after three time washes of pre-warmed DPBS.
4. (For positive control) Incubate cells with freshly prepared 0.1 mM H_2O_2 from 1 M stock in DPBS for 20 min at 37 °C after three time washes of pre-warmed DPBS.
5. Dilute the H_2DCFDA stock solutions into DPBS to make 0.1 μM working solution.
6. Cells were harvested by trypsinization.
7. Suspend cells in working solution at a density of 1×10^6 cells/ml and incubate at 37 °C for 30 min and protect from light.
8. Centrifuge the tubes at $130 \times g$ for 5 min.
9. Remove the supernatant and gently resuspend the cells in pre-warmed DPBS.
10. Repeat the wash steps 8 and 9 twice.
11. Submit samples to flow cytometry for ROS detection using the 488nm laser for excitation and detected at 535 nm.

Analysis

1. Gate on the main cell population.

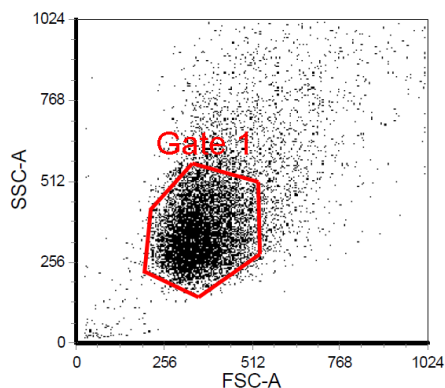


Figure 1. Cells were analyzed according to their size and granularity. The X-axis represents the forward scatter (FSC) parameter which is relative to the size for the cell. The Y-axis shows the side scatter (SSC) parameter which correlates with the components inside the cell. Gate 1 indicates the main population of the cells we analyzed.

2. Show the intensity of H₂DCFDA of cells in gate 1.

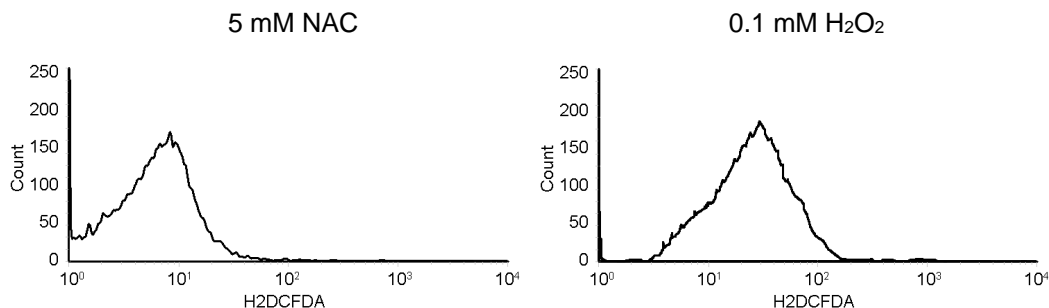


Figure 2. Histogram of H₂DCFDA. It shows how many cells are at each intensity of H₂DCFDA. The X-axis represents the H₂DCFDA intensity, while the Y-axis indicates the cell counts in corresponding fluorescence intensity.

Recipes

1. DPBS (1 L)
 - 8 g Sodium Chloride (NaCl)
 - 0.2 g Potassium Chloride (KCl)
 - 0.2 g Potassium Phosphate, monobasic (KH₂PO₄)

- 1.15 g Sodium Phosphate, dibasic (Na_2HPO_4)
Adjust to pH = 7.3.
2. H_2DCFDA stock (10 mM)
Dissolve 10 mg in 2.05 ml DMF to make 10 mM stock.
Aliquot and store at -20°C .
Avoid from light and repeated freeze/thaw cycles.
3. NAC stock (1 M)
Dissolve 1 g in 6.128 ml distilled water to make 1 M stock.
Filter, aliquot and store at -20°C .
Avoid from light and repeated freeze/thaw cycles.
4. H_2O_2 stock (1M)
Dilute 50 μl H_2O_2 from 35% (=11.6M) in 530 μl sterile deionized water to make 1 M stock.

References

1. Chang, H. Y., Huang, H. C., Huang, T. C., Yang, P. C., Wang, Y. C. and Juan, H. F. (2012). [Ectopic ATP synthase blockade suppresses lung adenocarcinoma growth by activating the unfolded protein response](#). *Cancer Res* 72(18): 4696-4706.